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Unexpected survival of mice carrying a mutation in Pygo2 that strongly reduces its binding to Bcl9/9l

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Abstract

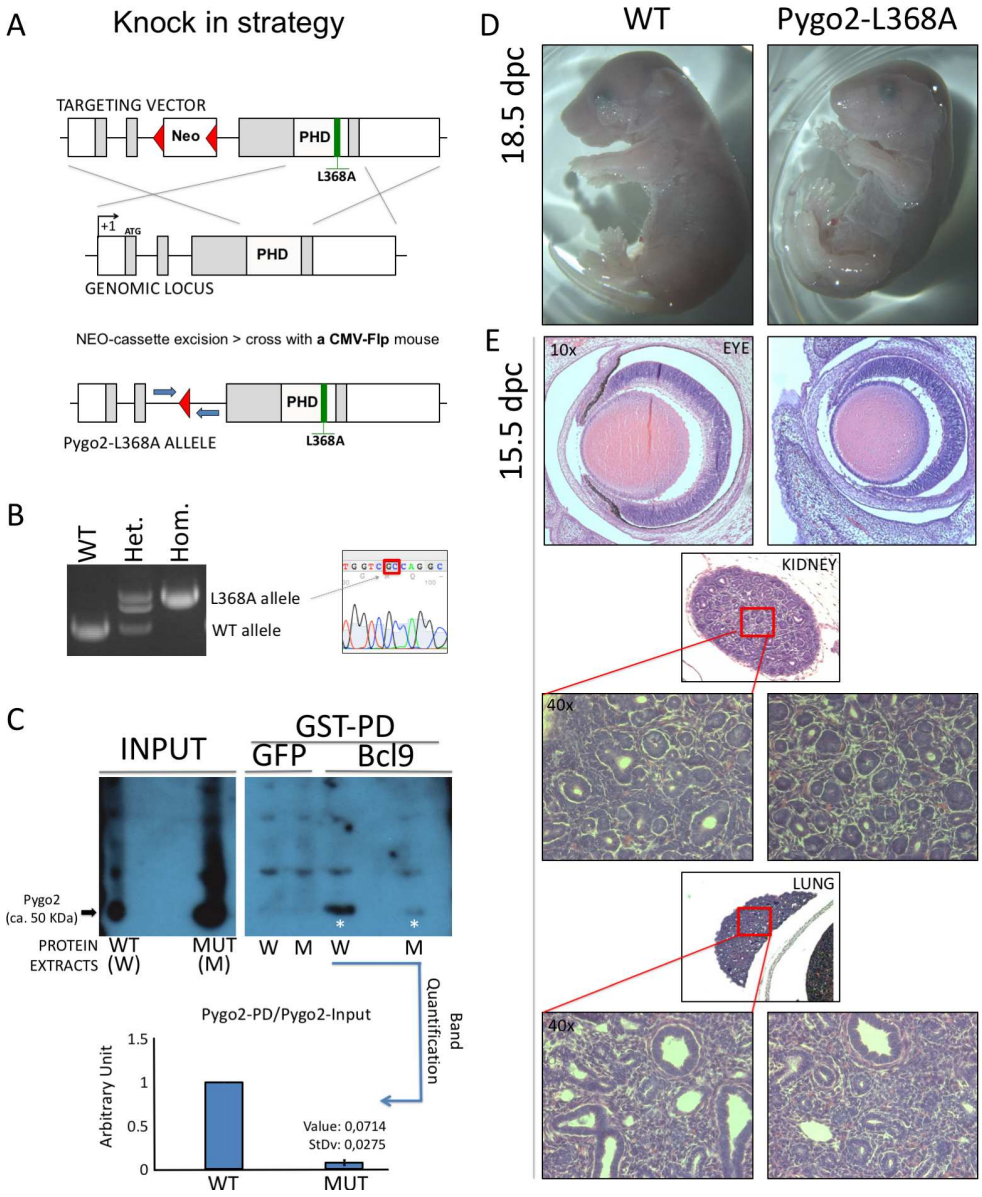
Pygopus is a transcriptional activator important for the Wnt signaling pathway. It binds to the beta-catenin transcriptional complex via the adaptor proteins Bcl9 and Bcl9l (Bcl9/9l). This complex is considered to be a suitable target for the treatment of tumors that display activated Wnt signaling. In the mouse, there are two Pygopus-encoding genes, *Pygo1* and *Pygo2* (*Pygo1/2*), with the latter playing a major role. Here we introduce a single amino acid substitution in Pygo2, which was previously shown to abrogate binding to Bcl9/9l, and cause lethality in *Drosophila melanogaster*. We confirm that mutant Pygo2 protein fails in interacting with Bcl9 but, unexpectedly, homozygous mice with this mutation are viable and fertile, even when this mutant allele is combined with a null mutation of the potentially redundant *Pygo1*. Based on this observation, we conjecture that the Pygo-Bcl9/9l interaction requires scant affinity *in vivo* to fulfill developmental functions and thrust forward the notion that this interaction surface could be targeted in cancer therapy without major consequences on homeostatic functions.

Objective

Our purpose is to further test the relevance of the Pygo2—Bcl9/9l interaction surface and the effect of its abrogation on mouse development. This interaction is a candidate target for the treatment of Wnt signaling-driven tumors.

Introduction

The Wnt signaling pathway drives virtually all aspects of embryonic development, and its over activation is a causative factor in several human cancers [1]. Beta-catenin is the molecular fulcrum of canonical Wnt signaling [2]. It binds to several transcriptional co-factors; among them, Bcl9/9l and Pygo1/2 [3] [4] [5]. Of note, while *Pygo1*-null mice are viable and fertile, mice deficient for *Pygo2* die between 13.5 days post coitum (dpc) and birth, presenting a series of developmental defects. Double *Pygo1/2* homozygous mutants do not display any synergy in the severity of the phenotype [6] [7]. Bcl9/9l act as "bridge" proteins by simultaneously binding beta-catenin (via the HD2 domain) and Pygo1/2 (via the HD1 domain). The relevance of this protein complex in the mouse is also supported by previous work: the deletion of the HD1 domain—which fully abrogates Bcl9/9l's ability to bind Pygo1/2—leads to embryonic lethality, recapitulating the complete loss of Pygo1/2 [8]. Earlier results identified the amino acids within the Plant Homology Domain (PHD) of Pygo proteins that are critical for binding to Bcl9/9l: these mutations (e.g., L789A) lead to embryonic lethality in *Drosophila melanogaster* [9]. Here we create the corresponding and functionally analogous mutation in the mouse *Pygo2*: the substitution of the Leucine in position 368 (L368A).



a

Figure Legend

Pygo2-L368A-mutant mice have no apparent phenotype.

(A) Schematic overview of the knock-in strategy used to generate the *Pygo2*-L368A-mutant allele. Boxes indicate exons, coding sequence is in grey. Exons and introns are not to scale. The L368 position within the Plant Homology Domain (PHD) is indicated by a green vertical bar. (B) Left: PCR genotyping of the Pygo2-L368A mice. Right: sequencing of the mutated allele using, as template, genomic DNA extracted from homozygous mice. (C) Top: GST-pulldown (GST-PD) experiment showing that the L368A mutation in the Pygo2 PHD finger efficiently reduces the Pygo2-Bcl9 binding. Note that a peptide spanning the N-terminal 372 amino acids of Bcl9 was used. Bottom: Fiji-based band quantification of three independent pull- down experiments-the bands obtained with the pull-down are quantified over their relative input, and the band intensity of the WT, for each experiment, is set to 1. The average value and the standard deviation (StDv) of the three quantifications are indicated. WT (W), wild-type; MUT (M), mutant. (D) Pygo2-L368A embryos at 18.5 dpc are normal in appearance. (E) There are no discernible developmental defects in *Pygo2*-L368A-mutant

embryos at 15.5 dpc. From top to bottom: hematoxylin/eosin staining of tissue sections from developing eye, kidney, and lung.

Results & Discussion

We generated a *Pygo2* knock-in mutant allele by introducing a two-nucleotide change (CT>GC) so that the Leucine in position 368—within the plant homology domain (PHD) of Pygo2—is replaced by an Alanine (Figure A). The homologous mutation in *pygo* (L789A) leads to lethality in *Drosophila melanogaster* [9]. The mice are genotyped with primers spanning the remaining FRT sequence that follows the NEO-cassette excision (Figure B, see Materials and Methods section for a detailed description). The presence of the mutation is confirmed by DNA sequencing (Figure B). Initially, three independent breedings between heterozygous mice ($Pygo2^{L368A/+}$ X $Pygo2^{L368A/+}$) gave rise to mutant homozygous $Pygo2^{L368A/L368A}$ animals (6/44 [13.6%], representing half of the expected Mendelian ratio). The homozygous mutant mice obtained were then interbred, giving rise to healthy animals, thereby showing that homozygous *Pygo2*-L368A mutant mice reach adulthood without any malformation and are fertile. To rule out any compensatory mechanism due to potential redundancy with Pygo1, we brought the *Pygo2*-L368A mutant allele into a *Pygo1*-knockout background. By crossing *Pygo2* heterozygous, *Pygo1*-knockout mice ($Pygo1^{-/-}$; $Pygo2^{L368A/+}$), we obtained *Pygo1/2* double mutant animals ($Pygo1^{-/-}$; $Pygo2^{L368A/L368A}$) in a proportion of 4/15 (26%—the expected Mendelian ratio is 1/4). A double mutant male mouse ($Pygo1^{-/-}$; $Pygo2^{L368A/368A}$) from this progeny was successively bred twice with a $Pygo1^{-/-}$; $Pygo2^{L368A/+}$ female and gave rise to a total of 10/23 double mutant mice (43.5%—the expected Mendelian ratio is 1/2). Both male and female double mutant mice could breed, allowing us to establish a colony of healthy $Pygo1^{-/-}$; $Pygo2^{L368A/L368A}$ animals.

The effect of the L368A mutation on the Pygo2—Bcl9 interaction was confirmed by *in vitro* GST pull-down experiments. We extracted proteins from adult wild-type and mutant kidneys, an abundant source for Pygo2 protein [10]. We incubated wild-type and mutant protein extracts with a recombinant GST-Bcl9 protein fragment (amino acids 1—372 of mouse Bcl9, that includes the region spanning two relevant domains: HD1 [Pygo-binding] and HD2 [beta-catenin-binding]). GST-GFP was used as negative control. With glutathione-conjugated sepharose beads, we pulled down the GST-proteins and performed Western blot analysis to detect Pygo2 in the pull-down reactions. Whereas the GST-Bcl9 can strongly interact with the wild-type Pygo2, it fails in significantly pulling down the Pygo2-L368A mutated protein (Figure C, compare the bands indicated by the two white asterisks).

Pygo1/2 double knockout mice die during embryonic development between 13.5 dpc and birth due to a series of developmental defects [11] [8]. However, Pygo2-L368A embryos are, in their superficial appearance, indistinguishable from wild-type littermates (Figure D). Out of 25 embryos analyzed, we scored six mutants (24%, close to the expected Mendelian ratio of 1/4). Moreover, histological analyses of the tissues affected during development in *Pygo1/2*-knockout mice (mainly the lens, the lungs, and the kidney [11] [6]) reveal no obvious alterations at 15.5 dpc (Figure E).

The complete abrogation of the interaction between Bcl9/9l and Pygo1/2—via the deletion of the HD1 domain in both Bcl9 and Bcl9l—leads to embryonic lethality at 13.5 dpc, with a striking "Pygo knockout" phenotype [8]. These results appear contradictory (see also the Alternative Explanations paragraph). It is possible that the deletion of the full Pygo-interacting HD1 domain of Bcl9/9l completely abrogates their interaction, while the single amino acid substitution L368A in Pygo2 strongly reduces their binding, but leaves some residual interaction. Thus, the residual binding between Pygo2-L368A and Bcl9 would be capable of fulfilling all the developmental functions for which this interaction is required. It is possible, in fact, that this interaction is weak and dynamic in nature *in vivo*. The extent to which the mutation L368A decreases the affinity between Pygo2 and Bcl9 remains to be determined. In conclusion, we cannot observe any developmental or homeostatic defect in mutant $Pygo1^{-/-}$; $Pygo2^{L368A/L368A}$ mice: they reach adulthood healthy and fertile. Because several reports have previously shown that the interaction between Pygo1/2 and Bcl9/9l is necessary for proper development [9] [12] [8], we propose that only a weak interaction between these factors is required.

Pygo2-L368A mice are viable and fertile despite displaying strongly reduced Pygo2-Bcl9/9l binding.
The main limitation of this study consists in the fact that, by using an *in vitro* binding assay to demonstrate the decreased affinity between the mutant Pygo2 and Bcl9, we cannot exclude that this interaction is intact in the cell.
Pygo2 overexpression has been shown to drive elevated Wnt signaling as potential causative factor in different types of tumors [12] [13] [14] [15]. It will be necessary to test whether the decreased affinity between Pygo2-L368A and Bcl9/9l is mirrored by an attenuated Wnt signaling. We conjecture that this mouse model could serve as an ideal tool to test, as proof of principle, if the Bcl9/9l-interacting surface of Pygo proteins could constitute the target of small compounds/inhibitors aimed at dampening the aberrantly activated Wnt signaling in these tumors.

Additional Information

Methods and Supplementary Material

Please see https://sciencematters.io/articles/201604000006.

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Ethics Statement

The mouse experiments were performed in accordance with Swiss guidelines and approved by the Veterinarian Office of the Canton of Zurich, Switzerland.

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